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Moringa oleifera stem extract protect skin keratinocytes against oxidative stress injury by enhancement of antioxidant defense systems and activation of PPARα

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ABSTRACT

Oxidative stress is an important cause of skin injury induced by UVB radiation. *Moringa oleifera* also known as horseradish tree or drumstick tree, have multiple nutraceutical or pharmacological functions. However, whether *Moringa oleifera* protects skin against oxidative stress injury remains unknown. To investigate the effects of the ethanol extract of *Moringa oleifera* stem (MSE) on skin oxidative stress injury and its molecular mechanism, we first determined the effect of MSE on epidermal oxidative stress injury induced by H_2O_2 in keratinocytes (HaCaT cells) and by UVB-radiation in mice. Then we investigated the effect of MSE on the enhancement of antioxidant system and activation of PPARα *in vitro* and *in vivo*. Furthermore, the flavonoids compositions in MSE were assayed by high-performance liquid chromatography (HPLC), and then molecular docking study was used to assess the major component in MSE to activate PPARα. Our results indicate that MSE (100–400 μg/mL) protected the epidemic cell against oxidative stress injury *in vitro* and topical treatment with MSE cream (6%) inhibit UVB-induced oxidative stress injury in the epidermis of the mouse skin. PPARα activation is involved in the protective effect of MSE. HPLC assay and molecular docking study indicated that rutin might be the main component in MSE to activate PPARα. These results confirm that MSE exerts the protective effect on oxidative stress induced skin keratinocytes injury. Moreover, the protective effect of MSE is mediated by enhancement of antioxidant defense systems and activation of PPARα in skin keratinocytes.

1. Introduction

Skin is the largest organ of the body and the anatomical location of epidermal keratinocytes makes them vulnerable to solar ultraviolet (UV) radiation. UVB (280–320 nm) is mostly absorbed by the epidermis, and epidermal injury may lead to sunburns, premature skin aging, and even skin cancer [1]. Oxidative stress is one of the main reasons for UVB radiation induced skin injury. UVB promotes the generation of reactive oxygen species (ROS) such as the superoxide anion (O2·) and hydrogen peroxide (H_2O_2) [2]. Failure of the cells to restore their redox homeostasis may lead to an overproduction of ROS attacking cellular lipids, proteins and DNA, leading to cell death. Therefore, the addition of antioxidants to skin care products has become a widely used practice. In particular, plant-derived antioxidants have drawn much attention for their low toxicity and high biological activity levels [3,4].

Moringa oleifera, also known as horseradish tree or drumstick tree, belongs to the *Moringaceae* family. All parts of the *Moringa oleifera* plant, such as its leaves, stems, roots, flowers, and seeds, have multiple nutraceutical or pharmacological functions such as anti-inflammatory, antioxidant, anti-cancer, hypoglycemic, and blood lipid-reducing functions [5–7]. Recently, the effect of *Moringa oleifera* on the skin has captured wide interest. Indeed, *Moringa oleifera* is reported to accelerate wound healing *in vitro* [8] and *in vivo* [9]. A recent study indicates that a cream formulation containing *Moringa oleifera* extract leads to skin revitalization in humans [10]. However, the effect of *Moringa oleifera* on skin epidermis after oxidative stress injury especially induced by UVB radiation still remains unknown. In addition, the molecular

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mechanism underlying its protective effect also needs to be clarified.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors and have three subtypes: PPARα, PPARβ and PPARγ. In skin, all three isoforms are present and PPARs have been regarded as a promising drug target for the treatment of cutaneous disorders [11]. Recently some researches indicate that PPARα may play an important role in keeping epidermal homeostasis against oxidative stress [12,13], and some natural PPARα agonists such as Eupatilin [14] and Sargahydroquinoic acid [15] have beneficial effects on human skin homeostasis. PPARα expression in mouse skin decreases after UV irradiation [13]. In addition, many total extracts of natural plants, such as the extracts of the *Cucurbita moschata* stems and *Camellia sinensis* leaves, have been reported to modulate PPARα activity [16]. Therefore, it is valuable to evaluate the effect of *Moringa oleifera* stem on PPARα activity.

Therefore, in this study, we first determined the effect of the ethanol extract of *Moringa oleifera* stem (MSE) on epidermal oxidative stress injury induced by H_2O_2 in keratinocytes (HaCaT cells) and by UVB-radiation in mice. Then we investigated the effect of MSE on the stimulation of antioxidant system and activation of PPARα *in vitro* and *in vivo*. Furthermore, the flavonoid compositions in MSE were assayed by high-performance liquid chromatography (HPLC), and then molecular docking study was used to predict the major component in MSE to activate PPARα.

2. Materials and methods

2.1. Reagents and materials

Polyethylene glycol (PEG) 8000, PEG 400, Dulbecco's Modified Eagle's Medium (DMEM), minimal essential medium (MEM), rutin, luteolin, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit was purchased from Promega Corporation (Madison, WI, USA). A lactate dehydrogenase (LDH) cytotoxicity assay kit, Hoechst 33258, and propidium iodide (PI) were purchased from Beyotime Biotechnology (Haimen, China). 2', 7'-Dichlorofluoresceindiacetate (DCFH-DA) and radioimmunoprecipitation assay (RIPA) lysis buffer were purchased from Solarbio (Beijing, China). Wy14643 and MK886 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). All other chemicals and reagents were of analytical grade.

2.2. Plant materials and preparation of MSE

Dry *Moringa oleifera* stems were obtained from Xiamen Jinzhu Ecological Agriculture Co., Ltd. (Fujian Province, China) and were identified by one of the authors (Dr. Chenqin), an expert from the School of Pharmaceutical Science of the Xiamen University (Xiamen, China). The dry *Moringa oleifera* stems (30 g) were ground into a fine powder and extracted with 70% ethanol (480 mL) at 85 °C for 2 h. Then, the insoluble mass was removed using Whatman filter paper no. 1, and the filtrate was dried using a rotary evaporator. The extracts were then lyophilized and stored at 4 °C. This extract was named MSE. For the *in vitro* study, MSE was freshly dissolved in MEM and then was filtered by 0.22 μM microfiltration membranes. For the *in vivo* study, according to our preliminary experiment, 1%, 6% MSE were chose for the formal experiments. MSE (1%, 6%) were prepared by dissolving MSE (0.01 g and 0.06 g) into 1 mL PEG cream (1:2 mixtures of PEG 8000 and PEG 400). Control cream was prepared by emulsifying distilled water into twice the volume of PEG cream.

2.3. HaCaT cell culture and oxidative stress injury induced by H_2O_2

The immortalized human skin keratinocytes cell line HaCaT was obstained from Kunming Cell Bank of the Chinese Academy of Sciences (KCB200442YJ, Kunming, China). HaCaT cells were cultured in MEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) and maintained at 37 °C in a 5% CO2 incubator. Oxidative stress is an important cause of skin aging. H_2O_2 is one of the major sources of ROS and has been extensively used to induce oxidative stress injury in HaCaT cells [17–19]. Our previous results indicated that treatment with 250 μM H_2O_2 for 24 h could induce oxidative stress injury in HaCaT cells (Fig. S1), so in this research, HaCaT cells were pretreated with MSE (50–400 μg/mL), rutin (25–200 μM) or Wy14643(100 μM) for 24 h, followed by incubation with H_2O_2 (250 μM) for another 24 h. In some research, the HaCaT cells were first pretreated with MK886 (5 μM) for 30 min and then treated with MSE (400 μg/mL), rutin (200 μM), or Wy14643 (100 μM) for 24 h, followed by incubation with H_2O_2 (250 μM) for another 24 h.

2.4. Cell viability and integrity assays in HaCaT cell

HaCaT cells viability was analyzed using the MTS assay. Briefly, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Then, the MTS working solution was added and the cells were incubated at 37 °C for 1 h. Absorbance was measured at 490 nm using a microplate reader (Varioskan, Thermo, USA).The results were expressed as a percentage of the control cell values.

HaCaT cell integrity was assessed using the LDH assay. Briefly, the cell culture supernatants were collected at the indicated times, and the intact cells were lysed using a solution of PBS (0.1 M, pH 7.4) containing 1% Triton X-100.The amount of LDH in the culture medium and lysate was determined spectrophotometrically at an absorbance of 490 nm. The released LDH was represented by the percentage of LDH in the culture medium relative to the total LDH. Cell morphology was examined, and the cells were photographed using an inverted microscope (Eclipse TS 100, Nikon, Tokyo, Japan).

2.5. Detection of apoptosis and necrosis in HaCaT cell

To further confirm the death of HaCaT cells after H_2O_2 (250 μM) exposure, we determined the levels of cell apoptosis and necrosis using a fluorescence double staining method with Hoechst 33258 and PI. After the H_2O_2 treatment for 24 h, the medium was removed, and the cells were washed twice with PBS and incubated with Hoechst 33258 (10 μg/mL) and PI (10 μg/mL) for 10 min at 37 °C. The cells were imaged using a fluorescence microscope (Ti-S, Nikon, Tokyo, Japan) under a 200 X field. Apoptotic cells were characterized by nuclear condensation and fragmentation after Hoechst 33258 staining, while PI stained necrotic cells red. One thousand cells were randomly selected, and the number of apoptotic or necrotic cells was counted according to a previously reported method [20]. Finally, the apoptotic and necrotic rates were calculated according to the following formula:

Apoptotic (necrotic) rate = the number of apoptotic (necrotic) cells/total number of counted cells × 100.

2.6. Measurement of intracellular ROS generation in HaCaT cell

The fluorescent probe DCFH-DA was used to detect intracellular ROS levels in HaCaT cells treated with MSE (50–400 μg/mL) alone or pre-treated with MSE before H_2O_2 exposure. For intracellular ROS assay, the cells were incubated with 25 μM DCFH-DA at 37 °C for 30 min in the dark and then imaged with a fluorescence microscope under a 200 X field. For the quantification of ROS generation, the cells were scraped in 1% Triton X-100, and then, the amount of DCF